

2018

Netrin-1-like-immunoreactivity co-expresses with DCC and has a differential level in the myenteric cholinergic and nitrergic neurons of the adult mouse colon

Suh Youn Ko

John T. Price

Gregory L. Blatch

The University of Notre Dame Australia, greg.blatch@nd.edu.au

Kulmira Nurgali

Follow this and additional works at: https://researchonline.nd.edu.au/health_article



Part of the [Medicine and Health Sciences Commons](#)

This article was originally published as:

Ko, S. Y., Price, J. T., Blatch, G. L., & Nurgali, K. (2018). Netrin-1-like-immunoreactivity co-expresses with DCC and has a differential level in the myenteric cholinergic and nitrergic neurons of the adult mouse colon. *Journal of Histochemistry and Cytochemistry*, Early View (Online First).

Original article available here:

[10.1369/0022155418819821](https://doi.org/10.1369/0022155418819821)

This article is posted on ResearchOnline@ND at https://researchonline.nd.edu.au/health_article/255. For more information, please contact researchonline@nd.edu.au.



This is the author's version of an article published in the *Journal of Histochemistry and Cytochemistry*, 21 December 2018, available online at
<https://journals.sagepub.com/doi/10.1369/002215541881982>

Ko, S.Y., Price, J.T., Blatch, G.L., and Nurgali, K. (2018) Netrin-1-like-immunoreactivity coexpresses with DCC and has a differential level in the myenteric cholinergic and nitrergic neurons of the adult mouse colon. *Journal of Histochemistry and Cytochemistry, Early View Online First*. doi: 10.1369/0022155418819821

Title: Netrin-1-like-immunoreactivity co-expresses with DCC and has a differential level in the myenteric cholinergic and nitrergic neurons of the adult mouse colon

Authors: Suh Youn Ko¹, John T. Price^{1,2,3,4}, Gregory L. Blatch^{1,5,6}, and Kulmira Nurgali^{1,3,4,#}

Institutional affiliations:

¹College of Health and Biomedicine, Institute for Health and Sport, Victoria University, Melbourne, Australia

²Department of Biochemistry and Molecular Biology, Monash University, Clayton, VIC, Australia

³Australian Institute for Musculoskeletal Science (AIMSS), Victoria University and University of Melbourne, Melbourne, VIC, Australia

⁴Department of Medicine Western Health, Faculty of Medicine, Dentistry and Health Sciences, The University of Melbourne, Melbourne, Australia

⁵The Vice Chancellery, The University of Notre Dame Australia, Fremantle, Western Australia, Australia

⁶Biomedical Biotechnology Research Unit (BioBRU), Department of Biochemistry and Microbiology, Rhodes University, Grahamstown, South Africa

Running headline/short title: Netrin-1 in the myenteric plexus

#Corresponding author:

Associate Professor Kulmira Nurgali, MBBS, MSc, PhD

College of Health and Biomedicine, Victoria University

Western Centre for Health, Research and Education

176 Furlong Road, St Albans, 3021, VIC, Australia

Tel: +61 (03) 8395 8223

Email: kulmira.nurgali@vu.edu.au

Abstract

Netrin-1 is a potent axonal and neuronal guidance cue in the developing nervous system. Netrin-1 functions are mediated by its receptors, such as deleted in colorectal cancer (DCC) present on axons and neurons. Localisation of DCC and Netrin-1 on various types of enteric neurons and their role in the mature enteric nervous system is unknown.

The results of our study revealed that almost all enteric neurons and processes express DCC and Netrin-1 in the adult mice. Netrin-1-like immunoreactivity (IR) was detected in the cytoplasm of neurons with some showing strong or weak staining.

The majority of Netrin-1-like immunoreactive enteric neurons were choline acetyltransferase (ChAT)-positive. However, ~19% of neurons were strongly Netrin-1-like-positive but ChAT-negative while ~8% of neurons were Netrin-1-like-negative but strongly ChAT-positive. In contrast, almost all nitric oxide synthase (nNOS)-positive enteric neurons displayed strong Netrin-1-like-IR. This differential intensity of Netrin-1 expression in the myenteric neurons might determine major neuronal subtypes regulating intestinal motility, ChAT-IR excitatory and nNOS-IR inhibitory muscle motor and interneurons.

This is the first study demonstrating the localisation of DCC and Netrin-1 in the colonic myenteric plexus of the adult mice and their expression level determining two major neuronal subtypes regulating intestinal motility.

Keywords: enteric nervous system (ENS), Netrin-1, deleted in colorectal cancer (DCC), choline acetyltransferase (ChAT), neuronal nitric oxide synthase (nNOS)

Introduction

Netrin-1 is a laminin-related protein that was initially discovered by examining diffusible cues secreted by the floor plate cells during spinal cord development [1]. Since then it has been known as a potent axonal and neuronal guidance cue in the developing central nervous system (CNS) [2].

The hallmark of Netrin-1 function is that it serves as either a chemoattractant or chemorepellent for different types of axons and neurons [3, 4]. These opposing roles of Netrin-1 are mediated by a particular type of Netrin-1 receptors, such as deleted in colorectal cancer (DCC) and UNC5 homologues (UNC5H), present on axons and neurons [5]. For example, DCC expressing axons elicit chemoattractive activity towards Netrin-1 molecules. On the other hand, UNC5H expressing axons mediate chemorepulsive activity in the presence of Netrin-1. This dynamic expression of Netrin-1 receptors on axons and neurons is directed to find their appropriate targets or prevented from aberrantly growing under Netrin-1 guidance [6].

In addition to the expression of Netrin-1 and its receptors and their bi-functional roles in the developing CNS, the expression of DCC in the developing enteric nervous system (ENS) has been noted since early 2000 [7]. Seaman *et al.* have demonstrated that DCC expression is present in the peripheral nervous system (PNS) of the developing mouse, in particular, at the early phase of development of the ENS [7]. This finding highlighted that DCC signalling is not limited to the CNS.

Subsequent studies have described that vagal neural crest-derived precursors, which colonise the gut and form the ENS, were found to express DCC [8]. During the colonisation, precursors initially establish the myenteric plexus and then the subset of those cells undergo a perpendicular secondary migration toward mucosa to form the

submucosal plexus and pancreatic ganglia. The migration of this subset was guided by Netrin-1 that originated from the mucosal layer of the gut and pancreas [8]. In addition to vagal neural crest-derived precursors, vagal sensory axons were found to up-regulate the expression of DCC at a certain developmental period, so that the sensory afferent innervation to the fetal gut could be mediated by Netrin-1 guidance [9]. It was further demonstrated that the sensory afferent innervations to the gut were only possible in the ganglionic regions, whereas no progressive innervations were made when the fibers reached the aganglionic regions.

Importantly, *in vitro* studies have revealed that the enteric neural crest-derived cells (ENCC) do not express Netrin-1, but they synthesize Netrin-1 once they give rise to enteric neurons [10]. Therefore, DCC-expressing vagal sensory afferent fibers were able to connect with the intrinsic enteric neurons expressing Netrin-1 [10].

The intrinsic nerve circuits of the ENS contain 14-20 types of enteric neurons depending on species and the region of the gut [11]. A vast number of neurotransmitters and neuropeptides have been identified in enteric neurons at different stages of the development [12]. The neurochemical coding of the enteric neurons has revealed a useful distinction between the enteric neurons in relation to their functional types [11]. As consequence, diverse subtypes of enteric neurons have been generated based on neurochemical phenotypes.

Certain neurochemical codes have been highly conserved between species. The conserved chemical codes are mostly for excitatory and inhibitory muscle motor neurons that are essential for gut motility. These include choline acetyltransferase (ChAT, an enzyme synthesizing acetylcholine) for excitatory muscle motor neurons

and interneurons [13], and neuronal nitric oxide synthase (nNOS, an enzyme synthesising nitric oxide) for inhibitory muscle motor neurons and interneurons [14].

It has been indicated that DCC/Netrin-1 signalling plays a pivotal role in establishing the ENS during development, and subsequent studies have indicated that Netrin-1 expression is present in the neural unit of the adult gut [15]. However, the localised expression of DCC and Netrin-1 and the role of Netrin-1 in the mature ENS have not been fully unravelled.

This study aimed to determine the localisation of DCC and Netrin-1 expression in the various types of colonic myenteric neurons of the healthy adult mice.

Materials and Methods

Animals

Male Balb/c mice aged 6-10 weeks (18-25 g) were purchased from the Animal Resources Centre (WA, Australia). Mice were housed in a controlled environment where autoclaved cages, bedding, food and water were provided. They were maintained on a daily 12-hour dark and light cycle at 22°C. Mice were acclimatised for a minimum of 3 days prior to commencing any experiments. All surgical and experimental procedures were conducted in accordance with the guidelines approved by the Victoria University Animal Experimentation Ethics Committee (AEETH21/11).

Wholemout immunofluorescence

The distal colon segments were collected from mice. Samples were placed in a phosphate buffered saline (PBS) solution (pH 7.2) containing an L-type calcium channel blocker, nifedipine (3 μ M), and supplied with carbogen for 20 min in order to hinder smooth muscle contraction. Samples were cut open along the mesenteric border, maximally stretched and pinned into a silicon coated dish after clearing the bowel contents. Tissues were fixed in a Zamboni's fixative (2% formaldehyde and 0.2% picric acid) solution at 4°C overnight. Tissues were then washed for 3 x 10 min in dimethyl sulfoxide (Sigma-Aldrich, Australia) followed by 3 x 10 min in PBS to remove fixative. Subsequently tissues were dissected to expose myenteric plexus and they were incubated with 10% donkey serum (Merck Millipore, USA) diluted in 0.1% PBS-tween 20 for 1 hour at room temperature (RT). The myenteric plexus in wholemount preparations were incubated overnight at 4°C in the primary antibodies diluents (listed in **Table 1**). When double- or triple-labelling was conducted in the wholemount preparations, the primary antibodies that were raised in different species were used. Following 3 x 10 min washes in a 0.1% PBS-tween 20 solution, the wholemount preparations were incubated for 1 hour at RT in the species-specific secondary antibodies labelled with different fluorophores (listed in **Table 2**). The wholemount preparations were washed 3 x 10 min in PBS-tween 20 (0.1%). Before the final washing step, the fluorescent nucleic acid stain 4'-6-diamidino-2-phenylindole (DAPI, 14 nM, D1306; ThermoFisher, Australia) was introduced to the wholemount preparation for 1 min, and they were mounted onto glass slides with fluorescent mounting medium (DAKO).

For secondary antibody control, myenteric plexus in wholemount preparations were incubated in a single primary antibody diluent, namely a chicken anti-neurofilament

heavy chain (NF-H) antibody at 4° overnight. Either anti-DCC or anti-Netrin-1 antibody was omitted in the primary antibody incubation whilst corresponding species-specific secondary antibodies were added during the secondary antibody incubation for 1 hour at RT (**Supplementary Fig. 1**). In addition, absorption control was conducted using Netrin-1 blocking peptide (Catalog number X1868b, Exalpha Biologicals) [16]. The wholemount preparations were incubated in the diluents of anti-NF-H antibody and the pre-absorbed Netrin-1 antibody overnight at 4°C. After washing, the wholemount preparations were incubated for 1 hour at RT in the species-specific secondary antibodies labelled with different fluorophores: donkey anti-chicken FITC and donkey anti-rabbit rhodamine (**Table 2**). Pre-absorbed Netrin-1 antibody was produced by mixing anti-Netrin-1 antibody (200 µg/mL) with Netrin-1 blocking peptide (1 mg/mL), at a working dilution of 1:10 (antibody: immunogen) ratio, and the mixture was incubated overnight at 4°C with a gentle agitation. A double-labelling of NF-H and Netrin-1 was conducted parallel to the absorption control (**Supplementary Fig. 2**). The immunoblot control of DCC antibody specificity was performed in SW480 cells transfected with plasmids including Neo-Bam (empty vector plasmid), DCC-Mutant (DCC gene missing entire intracytoplasmic domain), and DCC-Full (DCC gene full length). The specificity of DCC antibody was also confirmed by using Basic Local Alignment Search Tool (BLAST) (**Supplementary Fig. 3**).

In addition to using Netrin-1 and DCC antibodies from Santa Cruz Biotechnology, the wholemount preparations of the colon tissues were also labelled with antibodies from different sources: a chicken polyclonal anti-Netrin-1 antibody (Aves Labs, Cat #: NET), as well as a rabbit polyclonal anti-DCC antibody (Abnova, Cat #: PAB17539) (**Supplementary Fig. 4**). Since no differences in the pattern of Netrin-1-like-IR and

DCC IR was also observed by using these additional antibodies, the antibodies from Santa Cruz Biotechnology (listed in **Table 1**) were used in this study.

Imaging

The images of wholemount preparations were taken using an Eclipse Ti confocal laser scanning microscope (Nikon, Lidcombe, Australia) with NIS element software. Fluorophores were visualised using a 488 nm excitation filter for fluorescein isothiocyanate (FITC), a 561 nm excitation filter for Alexa 594, a 405 nm excitation filter for DAPI, and a 640 nm excitation filter for Alexa 647. Three-dimensional (Z-series) images of the wholemount preparations were acquired at a normal thickness of 0.5 μm , with 20x (dry, 0.75) or 40x (oil immersion, 1.3) or 100x (oil immersion, 1.45) objective lenses.

Neural cell counting and quantitative analysis

The immunoreactive neuronal cell counting was performed according to the method published previously [17]. The IR of DCC, Netrin-1-like, choline acetyltransferase (ChAT) and neuronal nitric oxide synthase (nNOS) positive neurons was assessed against IR of β -Tubulin III positive neurons in the myenteric plexus in total area of 2mm². Eight randomly chosen images per preparation were taken with a 20x objective. All acquired images were captured with an identical acquisition exposure setting, and they were analysed blindly. Thresholding for each channel was determined based on a single fluorophore labelling, and the calibrated threshold was applied to analyse double-labelled preparations. The strong and weak intensity of immunostaining was distinguished by setting a constant threshold using MatLab. In the case of Netrin-1 and ChAT double labelling, the merge images were threshold by

subtracting one channel from the other channel. As a result, the images show strongly stained neurons, but remove weakly stained neurons. In the case of Netrin-1 and nNOS double labelling, the threshold for Netrin-1 channel based on histogram settings at a constant value: channel Min = 20 to channel Max = 70. This allows us to collect the images showing neurons expressing a strong Netrin-1-like IR. The immunoreactive neurons were counted manually per ganglion in images (512 x 512 pixels per image) using ImageJ plugins cell counter tool (average 30 ganglia in total area of 2mm² per preparation).

Statistical analysis

Results are presented as mean \pm SD from 3 to 4 animals. GraphPad Prism 7 software was used for statistical analysis. One-way ANOVA followed by Tukey's multiple comparisons test or Kruskal-Wallis and Dunn's multiple comparison tests; two-way ANOVA followed by Tukey's multiple comparisons test were used; *p* value less than 0.05 was considered significant.

All raw data can be provided upon requests.

Results

Localisation of Netrin-1-like and DCC immunoreactivity in the myenteric plexus of the colon from healthy adult mice

In order to investigate whether or not Netrin-1 is expressed in the mature ENS, wholemount preparations of the adult mice colon tissues revealing the myenteric plexus were labelled with Netrin-1. Our results showed that the Netrin-1 is distinctively expressed in the mature myenteric plexus. The Netrin-1-like-IR was

found to be co-localised with a neuronal marker, protein gene product 9.5 (PGP9.5) that labels neuronal cell bodies and processes (**Fig. 1A**). Also, Netrin-1-like IR was clearly shown in the internodal strands forming a nerve plexus. This was detected by the co-localization of Netrin-1 staining combined with another neuronal marker specific to the NF-H (**Fig. 1B**). The Netrin-1-like-IR was primarily detected in the cytoplasm of the nerve cell bodies, and those Netrin-1-like positive neurons showed both strong and weak staining in their cell bodies. There were many punctate structures surrounding individual neurons within a ganglion (**Fig. 1C**). To see whether those punctate structures of Netrin-1-IR were associated with glial cells, intermediate filament glial fibrillary acidic protein (GFAP) was co-stained with Netrin-1. As expected, Netrin-1-like-positive neurons and processes were outlined by the GFAP-IR (**Fig. 1D**). However, the IR for GFAP and Netrin-1 markers did not appear to be co-localised (**Fig. 1E**). This suggests that Netrin-1 is specifically expressed in myenteric neurons.

Next, we examined whether or not Netrin-1 receptor, DCC expression is remained in the adult ENS. Our results revealed that DCC is also expressed in the mature myenteric plexus with a distinctively similar pattern to Netrin-1 expression. DCC staining was co-localised with the neuronal markers, PGP9.5 (**Fig. 2A**) and NF-H (**Fig. 2B**), suggesting that DCC expression is localised in neuronal cell bodies and nerve processes. Similar to Netrin-1 expression, DCC expression was shown in the cytoplasm of the neuronal cell bodies. A similar pattern of Netrin-1-like-IR and DCC IR was also observed by using additional antibodies from different sources (**Supplementary Fig. 4**). This supports the findings that Netrin-1 and DCC are present in the myenteric plexus of the colons in the adult mice. The expression of DCC was found to be evenly distributed among individual neurons (**Fig. 2C**). In

addition, co-labelling of DCC and GFAP was tested to see if this observed DCC expression was specific to the myenteric neurons or was shared with glial cells. The GFAP-IR was shown to outline the DCC-immunoreactive myenteric neurons and internodal strands (**Fig. 2D**). At a high magnification, some small punctuated structures were exhibited along where the glial cell fibers embraced the neurons (**Fig. 2E, arrowheads**). However, it appeared some glial cell fibers lack the DCC-IR (**Fig. 2E, arrows**). Collectively, these data suggested that DCC receptors were primarily expressed in myenteric neuronal cell bodies.

Since a similar pattern of the expression between Netrin-1 and DCC in the myenteric plexus was observed, a double-labelling of Netrin-1 and DCC was conducted to assess their co-localisation in the mature myenteric plexus (**Fig. 3A-C**). While DCC-IR was evenly distributed in individual neuronal cytoplasm within ganglia, it was evident that some neurons exhibited stronger Netrin-1-IR in their cytoplasm than others within a ganglion (**Fig. 3B**). Also, it revealed a strong IR of Netrin-1-like protein in the border of individual neurons (**Fig. 3B, B3'**) and the varicose axons (**Fig. 3C, C3'**). Taken together, these observations prompted us to investigate whether Netrin-1 expression is associated with certain neuronal subtypes. In the following section, the data concerning expression of Netrin-1 in relation to the expression of ChAT and nNOS in the colonic myenteric plexus are presented.

Co-expression of Netrin-1-like protein with ChAT and nNOS in the myenteric plexus of the colon from healthy adult mice

The motility of the gut is controlled by excitatory and inhibitory motor neurons, which are identified by neurochemical coding such as ChAT and nNOS, respectively [18,

19]. These ChAT and nNOS markers were used to test whether or not the Netrin-1-like-positive neurons overlap with either of those two markers in the myenteric plexus of the colon in healthy young adult mice. As discussed earlier, the Netrin-1-like-IR was found in the myenteric plexus, and this time it was indicated by showing the co-localisation of Netrin-1-like protein with a pan-neuronal marker, β -Tubulin III (**Fig. 4A**). Also, the IR of ChAT was shown to be co-localised with the β -Tubulin III marker, and the majority of myenteric neurons were labelled with the ChAT marker in the myenteric plexus (**Fig. 4B**). Similar to what had seen in the case of Netrin-1-like-positive neurons, there were either strongly or weakly labelled ChAT-positive neuronal cell bodies in ganglia. The co- labelling of Netrin-1 with ChAT was conducted in the myenteric plexus of the colon tissues to confirm their overlapping population (**Fig. 4C**). Although the majority of the Netrin-1-like-positive and ChAT-positive neurons were shown to overlap, 4 different combinations of stained neurons were observed and were indicated as arrows and arrowheads in **Fig. 4C, C1'-C3'**. These were grouped as neurons that were: (1) strongly Netrin-1-like-positive but ChAT-negative (N1++/ChAT-, filled arrowheads); (2) Netrin-1-like-negative but strongly ChAT-positive (N1-/ChAT++, empty arrowheads); (3) strongly Netrin-1-like-positive but also weakly positive for ChAT (N1++/ChAT+, filled arrows); (4) weakly Netrin-1-like-positive but also strongly positive for ChAT (N1+/ChAT++, empty arrows). The groups 3 and 4 were combined into one group, namely N1+(+)/ChAT+(+), since those neurons expressed both Netrin-1 and ChAT markers (**Fig. 4D**).

The quantified results showed that either weakly or strongly co-labelled neurons for both Netrin-1 and ChAT markers, N1+(+)/ChAT+(+), were found to be $73.3 \pm 3.1\%$. On the other hand, the non-overlapping population which means the neurons positive

only for either Netrin-1-like or ChAT marker were shown to be $26.7 \pm 3.1\%$. This included the population of N1⁺⁺/ChAT⁻ ($19.2 \pm 3.0\%$) and the population of N1⁻/ChAT⁺⁺ ($7.5 \pm 3.1\%$).

Next, in order to examine whether or not the strong Netrin-1-like-positive neurons overlap with nNOS-positive neurons in the myenteric plexus of the colon from healthy young adult mice, the nNOS marker was co-labelled with Netrin-1 marker (**Fig. 5C**). The result showed that the proportion of neurons, that were strongly positive for Netrin-1-like protein, was $43.1 \pm 3.8\%$ (**Fig. 5D**). The proportion of neurons that were positive for nNOS was $32.0 \pm 3.6\%$. Around 67% of neurons expressing strong Netrin-1-like protein were also positive for nNOS (N1⁺⁺/nNOS⁺), which is $30.2 \pm 4.5\%$ of the total number of neurons. This suggests that a high expression of Netrin-1 is associated with the nNOS phenotype in the myenteric plexus of the colon.

Discussion

Intrinsic expression of Netrin-1 and DCC in the colonic myenteric plexus of healthy adult mice

The work of Ratcliffe *et al.* [20] has indicated that the Netrin-1 receptor, DCC was found to express in the growth cones of the vagal sensory axons during the embryonic development of mouse gut. These axons were migrated toward the intrinsic enteric neurons where Netrin-1 was thought to be synthesised. Hence, a neural network between the CNS and the ENS is formed during development [9, 10]. However, the expression of DCC and Netrin-1 and their role in the mature ENS of the adult gut have not been elucidated.

To the best of our knowledge, this is the first study to present the localisation of DCC and Netrin-1 in the mature ENS. The immunofluorescence labelling of colonic wholemount preparations indicated that Netrin-1 and the chemoattractive Netrin-1 receptor, DCC, were expressed in the myenteric plexus of the colon in healthy adult mice. In addition to DCC, the chemorepulsive Netrin-1 receptor, UNC5H2, was also examined in our study. Although some immunoreactivity of UNC5H2 in myenteric ganglia was detected, the staining of UNC5H2 was rather indefinable (data not shown).

The vast majority of all myenteric neurons were positive for DCC and Netrin-1. Also, double-labelling of Netrin-1 and DCC showed that both markers were clearly co-localised in the cytoplasm of the neuronal cell bodies and processes.

Previously, the co-localisation of Netrin-1 and DCC was found in the cell bodies of neuronal precursor cells from the ventricular zone of the hippocampus in the developing murine brain. A decrease or increase in the level of Netrin-1 or DCC is found to affect neuronal precursor cell survival, regulation of neurogenesis and differentiation in developing murine hippocampus [21].

In the case of developing mammalian cerebral cortex, immunohistochemical labelling revealed that Netrin-1 and DCC localisation overlaps in the excitatory synapses during the development and the early postnatal period of synaptogenesis. It had been demonstrated that manipulating gain or loss of Netrin-1 function in the synapses resulted in regulation of the number and stabilisation of pre-synapse and post-synapse formation. The authors suggested that Netrin-1 is a potent synaptogenic molecule for cortical neuron excitatory synapse formation [22].

In the adult CNS, Netrin-1 expression is found in motor neurons and dorsal interneurons of the rodent spinal cord [23, 24]. Particularly, Netrin-1 expression is mostly enriched in periaxonal myelin membranes, and Netrin-1 is thought to serve as a short-range cue to maintain the appropriately formed network structure in the adult mammalian CNS [25, 26]. A short-range role of Netrin-1 can be mediated in cell-to-cell interactions [27]. Transient cell-to-cell interactions in the vertebrate olfactory system were shown to mediate patterning of neuronal connectivity by some cells acting as scaffolds for axon guidance and synaptogenesis [28].

Furthermore, in vitro studies have indicated that Netrin-1 can serve as an autocrine factor, and this promotes focal adhesions of the cells thereby slowing cell migration [29]. The autocrine and paracrine functions of Netrin-1 have been shown to promote osteoclast differentiation [30].

Our study demonstrates the co-localisation of Netrin-1-like protein and DCC expression in the mature myenteric plexus in the murine colon tissues. At this stage, we can only speculate that this co-localisation may be due to the consequence of neuronal precursor cell survival and/or differentiation. Ultimately, it may suggest a stabilisation of neuronal synaptic formation in the mature enteric neurons.

Determining the relationship of Netrin-1 and DCC co-expression in the mature enteric neurons would be greatly beneficial to understand a functional role of Netrin-1 in the adult ENS.

Particularly, our results showed that Netrin-1-like-positive neurons exhibited both strong and weak staining in their cytoplasm. This observation led us to examine whether or not there was any potential relationship between Netrin-1 expression with

specific subtypes of neurons, such as excitatory and inhibitory motor neurons and interneurons. This is discussed in more detail in the next section.

Moreover, some punctate structures from Netrin-1-like-IR were notably observed on the edges of the neuronal cell bodies and along the processes; possibly they are varicosities of the axons where neurotransmitters are contained and released [31]. Often successive varicosities are distributed along an axon [32]. Synaptophysin is a synaptic vesicle marker that has been found in myenteric ganglia and nerve fibers including varicosities in human and rat ENS [33]. The vesicular acetylcholine transporter (VACHT) is a neurotransmitter transporter that functions to load acetylcholine (ACh) into synaptic vesicles in the cleft [34]. Previous studies have shown synaptophysin and VACHT immunolabelling in enteric neurons which appear as granular and/or punctated structures within the neuronal fibers and enteric glial cells [35, 36], similar to Netrin-1-like-IR found in this study.

In recent years, Netrin-1 function has been found to be involved in nerve regeneration in the adult CNS and PNS [24, 37, 38]. Studies concerning middle cerebral artery occlusion (MCAO) have indicated that forced up-regulation of Netrin-1 in the peri-ischemic area of the adult rat's ischemic brain resulted in the up-regulation of DCC receptors [39]. The Netrin-1/DCC signalling pathway is found to activate the JNK1/c-Jun pathway that facilitates axonal regeneration and synaptic formation after the MCAO [39]. In particular, Netrin-1 has been implicated in neural plasticity and neuronal regeneration in the adult PNS following injury [37, 38]. The expression of Netrin-1 receptors such as DCC and UNC5B was significantly up-regulated in the injured sciatic nerve when compared to a normal sciatic nerve [37]. After sciatic nerve crush in rats, bone marrow mesenchymal stem cells (BMSCs) producing

Netrin-1 were transplanted and these cells showed a significant improvement of nerve recovery by promoting axon regeneration, when compared to the rats transplanted with BMSCs alone [37].

In our study, co-labelling of DCC or Netrin-1 and a glial cell marker, GFAP was tested to see whether or not glial cells also express DCC and/or Netrin-1. It appeared that the Netrin-1-like protein is specifically expressed by myenteric neurons, while the DCC is primarily expressed by myenteric neurons as well as some glial cells. GFAP was chosen because its expression is known to be rich in mature enteric glial cells [17, 40]. However, additional glial cell markers such as calcium binding protein, S100 β or transcription factor SRY box-containing gene 10, (SOX10) [41] may help to further clarify this question.

Correlation between Netrin-1 expression and the phenotypes of ChAT- and nNOS-positive neurons in the myenteric plexus

The ENS is composed of many different functional types of enteric neurons in mammals [18]. Motility of the gut is regulated by the neuronal circuitry of the myenteric plexus. The subtypes of myenteric neurons in the mouse have been characterised by neurochemistry and electrophysiology [42, 43]. The subtypes include inhibitory and excitatory motor neurons, ascending and descending interneurons and intrinsic sensory neurons. Among those, two major subtypes of myenteric neurons are involved in regulating motility: excitatory motor neurons and interneurons expressing ChAT and inhibitory motor neurons and interneurons expressing nNOS.

Here, we investigated the expression of ChAT and nNOS in relation to Netrin-1 expression in the myenteric neurons in adult healthy mice. It was revealed that the majority of Netrin-1-like expressing neurons also expressed ChAT. However, approximately 19% of neurons were strongly Netrin-1-like-positive but ChAT-negative. In contrast, a small population, around 8% of neurons, were Netrin-1-like-negative but strongly ChAT-positive.

In the case of nNOS-expressing subtype, almost all of nNOS-positive enteric neurons were found to also be strongly Netrin-1-like IR. This suggests that there is a prominent correlation between Netrin-1 and nNOS expression in the colonic myenteric plexus of the healthy adult mice.

A differential intensity of IR in the retinal ganglion cells has been monitored during normal development and after optic tract lesion in rats [44]. The authors noted that changes in the immunostaining pattern of ganglion cells were involved in retinal neuronal survival, differentiation and synaptogenesis.

Moreover, the correlation between cancer antigen (CA-125) expression and pathological parameters in endometrial carcinoma has been evaluated by immunohistochemistry [45]. It was noted that the intensity of positively labelled cells (i.e. strong or weak or negative labelling) is correlated with the stages of cancer invasiveness.

Our unpublished data revealed that the intensity of Netrin-1-like IR and ChAT was modulated in the myenteric plexus of the colon from mice with colorectal cancer. The contribution of Netrin-1 to phenotypic changes in enteric neurons in pathological conditions, such as cancer, may be worth investigating in the future.

Conclusion

Our results revealed that Netrin-1 and DCC are co-expressed in the mature myenteric plexus in the murine colon. This may suggest that Netrin-1 could serve as either short-range cue for cell-to-cell interactions or autocrine and paracrine functions for survival/differentiation and/or stabilising neural connectivity in the ENS. The direct relationship of Netrin-1 and DCC co-expression in the myenteric neurons needs to be elucidated in the future. In addition, the expression of Netrin-1 is found to be associated with major neuronal subtypes, such as ChAT and nNOS expressing myenteric neurons. Investigating the nerve regeneration role of Netrin-1 in the adult ENS under the pathological conditions involving an enteric nerve injury would be worth examining to find a potential therapeutic use of Netrin-1 in the adult ENS.

Acknowledgements

The authors would like to thank the animal technicians at Victoria University for their assistance in animal husbandry. Also, we thank Ms. Valentina Jovanovska and Dr. Aaron Harwood for assisting in imaging analysis. We acknowledge Biological Optical Microscopy Platform at the University of Melbourne for microscopic software training.

Competing Interests

We declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Author Contributions

SYK performed and analysed all experiments, and wrote the manuscript. KN designed the research and wrote the manuscript. The manuscript was reviewed and edited by KN, GB and JP. All authors read and approved the final manuscript.

Funding Statement

This research work was supported by the College of Health and Biomedicine at Victoria University.

Literature Cited

1. Serafini, T., Kennedy, T.E., Gaiko, M.J., Mirzayan, C, Jessell, T.M. and Tessier-Lavigne, M., *The netrins define a family of axon outgrowth-promoting proteins homologous to C. elegans UNC-6*. Cell, 1994. **78**(3): p. 409-424.
2. Sun, K.L.W., Correia, J.P., and Kennedy, T.E., *Netrins: versatile extracellular cues with diverse functions*. Development, 2011. **138**(11): p. 2153-2169.
3. Colamarino, S.A. and Tessier-Lavigne, M., *The axonal chemoattractant netrin-1 is also a chemorepellent for trochlear motor axons*. Cell, 1995. **81**(4): p. 621-629.
4. Kennedy, T.E., Wang, H., Marshall, W. and Tessier-Lavigne, M., *Axon Guidance by Diffusible Chemoattractants: A Gradient of Netrin Protein in the Developing Spinal Cord*. The Journal of Neuroscience, 2006. **26**(34): p. 8866-8874.
5. Webber, C.A., Christie, K.J., Cheng, C., Martinez, J.A., Singh, B., Singh, V., Thomas, D. and Zochodne, D.W., *Schwann cells direct peripheral nerve regeneration through the Netrin-1 receptors, DCC and Unc5H2*. Glia, 2011. **59**(10): p. 1503-1517.
6. Grunwald, I.C. and Klein, R., *Axon guidance: receptor complexes and signaling mechanisms*. 2002, Elsevier Science: Great Britain. p. 250.
7. Seaman, C., Anderson, R., Emery, B. and Cooper, H.M., *Localization of the netrin guidance receptor, DCC, in the developing peripheral and enteric nervous systems*. Mechanisms of Development, 2001. **103**(1-2): p. 173-175.
8. Jiang, Y., Liu, M.T, and Gershon, M.D., *Netrins and DCC in the guidance of migrating neural crest-derived cells in the developing bowel and pancreas*. Developmental Biology, 2003. **258**(2): p. 364-384.
9. Ratcliffe, E.M., Setru, S.U., Chen, J.J., Li, Z.S., Dautreaux, F. and Gershon, M.D., *Netrin/DCC-mediated attraction of vagal sensory axons to the fetal mouse gut*. The Journal of Comparative Neurology, 2006. **498**(5): p. 567-580.
10. Ratcliffe, E.M., Fan, L., Mohammed, T.J., Anderson, M., Chalazonitis, A. and Gershon, M.D., *Enteric neurons synthesize netrins and are essential for the development of the vagal sensory innervation of the fetal gut*. Developmental Neurobiology, 2011. **71**(5): p. 362-373.
11. Furness, J., *Enteric nervous system: neural circuits and chemical coding*. Encyclopedia of Neuroscience. Academic Press, Oxford, 2009: p. 1089-1095.
12. Hao, M.M. and Young, H.M., *Development of enteric neuron diversity*. Journal of Cellular and Molecular Medicine, 2009. **13**(7): p. 1193-1210.
13. Porter, A.J., Wattchow, D.A., Brookes, S.J.H. and Costa, M., *Cholinergic and nitrergic interneurons in the myenteric plexus of the human colon*. Gut, 2002. **51**(1): p. 70-75.
14. Tricoire, L. and Vitalis, T., *Neuronal nitric oxide synthase expressing neurons: a journey from birth to neuronal circuits*. Frontiers in Neural Circuits, 2012. **6**: p. 82.
15. Aherne, C.M., Collins, C.B., Masterson, J.C., Tizzano, M., Boyle, T.A., Westrich, J.A., Parnes, J.A., Furuta, G.T., Rivera-Nieves, J. and Eltzschig, H.K., *Neuronal guidance molecule netrin-1 attenuates inflammatory cell trafficking during acute experimental colitis*. Gut, 2012(5): p. 695.
16. Moon, C., Ahn, M., Jeong, C., Kim, H. and Shin, T., *Immunohistochemical study of netrin-1 in the spinal cord with rat experimental autoimmune encephalomyelitis*. Immunological Investigations, 2011. **40**(2): p. 160-171.

17. Robinson, A.M., Stojanovska, V., Rahman, A.A., McQuade, R.M., Senior, P.V. and Nurgali, K., *Effects of Oxaliplatin Treatment on the Enteric Glial Cells and Neurons in the Mouse Ileum*. Journal of Histochemistry and Cytochemistry, 2016. **64**(9): p. 530.
18. Furness, J.B., *Types of neurons in the enteric nervous system*. Journal of the Autonomic Nervous System, 2000. **81**(1-3): p. 87-96.
19. Sharrad, D.F., Chen, B.N. and Brookes, S.J.H., *Neurochemical coding compared between varicose axons and cell bodies of myenteric neurons in the guinea-pig ileum*. Neuroscience Letters, 2013. **534**: p. 171-176.
20. Ratcliffe, E.M., Farrar, N.R. and Fox, E.A., *Development of the vagal innervation of the gut: steering the wandering nerve*. Neurogastroenterology & Motility, 2011. **23**(10): p. 898-911.
21. Albright, C.D., Mar, M.H., Craciunescu, C.N., Song, J. and Zeisel, S.H., *Maternal dietary choline availability alters the balance of netrin-1 and DCC neuronal migration proteins in fetal mouse brain hippocampus*. 2005, Elsevier Science B.V., Amsterdam.: Netherlands. p. 149.
22. Goldman, J.S., Ashour, M.A., Magdesian, M.H., Tritsch, N.X., Harris, S.N., Christofi, N., Chemali, R., Stern, Yaakov E., Thompson-Steckel, Greta., Gris, Pavel., Glasgow, Stephen D., Grutter, Peter., Bouchard, Jean-Francois., R., Edward S., S, David. and Kennedy, T.E., *Netrin-1 Promotes Excitatory Synaptogenesis between Cortical Neurons by Initiating Synapse Assembly*. The Journal of Neuroscience, 2013. **33**(44): p. 17278-17289.
23. Manitt, C., Wang, D., Kennedy, T.E. and Howland, Dena R., *Positioned to inhibit: Netrin-1 and netrin receptor expression after spinal cord injury*. 2006, John Wiley & Sons, Ltd: United States. p. 1808.
24. Dun, X.-P. and Parkinson, D.B., *Role of Netrin-1 Signaling in Nerve Regeneration*. International Journal of Molecular Sciences, 2017. **18**(3): p. 491.
25. Manitt, C., Colicos, M.A., Thompson, K.M., Rousselle, E., Peterson, A. C. and Kennedy, T.E., *Widespread expression of netrin-1 by neurons and oligodendrocytes in the adult mammalian spinal cord*. The Journal Of Neuroscience: The Official Journal Of The Society For Neuroscience, 2001. **21**(11): p. 3911-3922.
26. Manitt, C. and Kennedy, T.E., *Chapter 32 Where the rubber meets the road: netrin expression and function in developing and adult nervous systems*. Progress in Brain Research, 2002. **137**: p. 425-442.
27. Nikolopoulos, S.N. and Giancotti, F.G., *Netrin-Integrin Signaling in Epithelial Morphogenesis, Axon Guidance and Vascular Patterning*. 2005, Landes Bioscience: United States. p. 429.
28. Chao, D.L., Ma, L. and Shen, K., *Transient cell-cell interactions in neural circuit formation*. Nature reviews. Neuroscience, 2009. **10**(4): p. 262-271.
29. Jarjour, A.A., Durko, M., Luk, T.L., Marçal, N., Shekarabi, M. and Kennedy, T.E., *Autocrine Netrin Function Inhibits Glioma Cell Motility and Promotes Focal Adhesion Formation*. PLoS ONE, 2011. **6**(9): p. 1-11.
30. Mediero, A., Ramkhalawon, B., Perez-Aso, M., Moore, K.J. and Cronstein, B.N., *Netrin-1 Is a Critical Autocrine/Paracrine Factor for Osteoclast Differentiation*. Journal of Bone & Mineral Research, 2015. **30**(5): p. 837-854.
31. Shepherd, G.M.G., Raastad, M. and Anderen, P., *General and variable features of varicosity spacing along unmyelinated axons in the hippocampus*

and cerebellum. Proceedings of the National Academy of Sciences of the United States of America, 2002. **99**(9): p. 6340-6345.

32. Gabella, G., *Fine structure of the myenteric plexus in the guinea-pig ileum*. Journal of Anatomy, 1972. **111**(Pt 1): p. 69-97.
33. Böttner, M., Harde, J., Barrenschee, M., Hellwig, I., Vogel, I., Ebsen, M. and Wedel, T., *GDNF induces synaptic vesicle markers in enteric neurons*. Neuroscience Research, 2013. **77**: p. 128-136.
34. Sugita, S., Wood, Caleb., Fleming, Leland L., Vaughan, Sydney K., Valdez, Gregorio, Gomes, M.P.S.M., Guatimosim, C., Camargo, W., Naves, L.A., Prado, V.F. and Prado, M.A.M., *VACHT overexpression increases acetylcholine at the synaptic cleft and accelerates aging of neuromuscular junctions*. Skeletal Muscle, 2016. **6**: p. 1-17.
35. Boesmans, W., Martens, M., Weltens, N., Hao, Marlene., Tack, J., Cirillo, C. and Vanden B.P., *Imaging neuron-glia interactions in the enteric nervous system*. Frontiers in Cellular Neuroscience, 2013. **7**(183).
36. Tong, Q. and Kirchgessner, A.L., *Localization and function of metabotropic glutamate receptor 8 in the enteric nervous system*. American Journal of Physiology - Gastrointestinal and Liver Physiology, 2003. **285**(5): p. G992-G1003.
37. Ke, X., Li, Q., Xu, L., Zhang, Y., Li, D., Ma, J. and Mao, X., *Netrin-1 overexpression in bone marrow mesenchymal stem cells promotes functional recovery in a rat model of peripheral nerve injury*. Journal Of Biomedical Research, 2015. **29**(5): p. 380-389.
38. Jaminet, P., Köhler, D., Schäufele, M., Rahmanian-Schwarz, A., Lotter, O., Fornaro, M., Ronchi, G., Geuna, S., Rosenberger, P. and Schaller, H-E., *Evaluating the role of Netrin-1 during the early phase of peripheral nerve regeneration using the mouse median nerve model*. Restorative Neurology And Neuroscience, 2013. **31**(3): p. 337-345.
39. Zheng, M., Chen, R., Chen, H., Zhang, Y., Chen, J., Lin, P., Lan, Q., Yuan, Q., Lai, Y., Jiang, X., Pan, X. and Liu, N., *Netrin-1 Promotes Synaptic Formation and Axonal Regeneration via JNK1/c-Jun Pathway after the Middle Cerebral Artery Occlusion*. Frontiers in Cellular Neuroscience, 2018. **12**(13).
40. Jessen, K.R. and Mirsky, R., *Glial cells in the enteric nervous system contain glial fibrillary acidic protein*. Nature, 1980. **286**(5774): p. 736-737.
41. Hao, M.M., Capoccia, E., Cirillo, C., Boesmans, W. and Vanden B.P., *Arundic Acid Prevents Developmental Upregulation of S100B Expression and Inhibits Enteric Glial Development*. Frontiers in Cellular Neuroscience, 2017. **11**(42).
42. Sang, Q. and Young, H.M., *Chemical coding of neurons in the myenteric plexus and external muscle of the small and large intestine of the mouse*. Cell And Tissue Research, 1996. **284**(1): p. 39-53.
43. Nurgali, K., Stebbing, M.J. and Furnes, J.B., *Correlation of electrophysiological and morphological characteristics of enteric neurons in the mouse colon*. The Journal Of Comparative Neurology, 2004. **468**(1): p. 112-124.
44. Chen, S.T., Patel, A.J., Garey, L.J. and Jen, L.S., *Expression of beta-amyloid precursor protein immunoreactivity in the retina of the rat during normal development and after neonatal optic tract lesion*. 1997. p. 713-717.
45. Metindir, J., Dilek, G.B. and Pak, I., *Staining characterization by immunohistochemistry of tumor cancer antigen in patients with endometrial cancer The correlation between pattern of CA-125 expression and pathologic*

parameters in endometrial carcinoma was evaluated. 2008, I.R.O.G. CANADA
INC.: Italy. p. 489.

Figure Legends

Figure 1. Localisation of Netrin-1 in the myenteric plexus of the colon from healthy young adult mice

The myenteric plexus of the colon tissues were stained with DAPI, nuclei staining (**A1-E1**), neuronal markers, PGP9.5 (**A2 and C2**) and NF-H (**B2**), as well as a glial marker, GFAP (**D2 and E2**). Netrin-1 (**A3-E3**) was labelled and the merged images (**A4-E4**) demonstrate the co-localisation of neuronal and glial markers with Netrin-1. N=5 animals. Scale bars: 100 μm (**A1-4, B1-4, D1-4**); 25 μm (**C1-4, E1-4**).

Abbreviations: PGP9.5, protein gene product 9.5; NF-H, neurofilament-heavy chain; GFAP, glial fibrillary acidic protein; DAPI, 4',6-diamidino-2-phenylindole.

Figure 2. Localisation of DCC receptors in the myenteric plexus of the colon from healthy young adult mice

The myenteric plexus of the colon tissues were stained with DAPI, nuclei staining (**A1-E1**), neuronal markers, PGP9.5 (**A2 and C2**) and NF-H (**B2**), as well as a glial marker, GFAP (**D2 and E2**). DCC receptors (**A3-E3**) were labelled and the merged images (**A4-E4**) demonstrate the co-localisation of neuronal and glial markers with DCC receptors. Filled arrowheads in **E2-4** indicate the co-localisation regions of GFAP and DCC markers, whilst arrows indicate lack of co-localisation of GFAP and DCC makers (**E2-4**). N=5 animals. Scale bars: 100 μm (**A1-4, B1-4, D1-4**); 25 μm (**C1-4, E1-4**).

Abbreviations: DCC, deleted in colorectal cancer; PGP9.5, protein gene product 9.5; NF-H, neurofilament-heavy chain; GFAP, glial fibrillary acidic protein; DAPI, 4',6-diamidino-2-phenylindole.

Figure 3. Co-localisation of DCC-IR and Netrin-1-IR in the myenteric plexus of the colon from healthy young adult mice

The myenteric plexus of colon tissues were stained with DAPI, nuclei staining (**A1-C1**), DCC receptors (**A2-C2**) and Netrin-1 (**A3-C3**). The merged images (**A4-C4**) demonstrate the co-localisation of DCC receptors and Netrin-1. The inserted box area of **B3** is shown with a high magnification view (**B3'**) and it highlights the punctated structure around the cell body. The arrows in **C3'** indicate the IR of Netrin-1 on varicosities. N=5 animals/group. Scale bars: 100 μm (**A1-4**); 25 μm (**B1-4**), 50 μm (**C1-4**).

Abbreviations: DCC, deleted in colorectal cancer; IR, immunoreactivity; DAPI, 4',6-diamidino-2-phenylindole.

Figure 4. Co-localisation of Netrin-1-IR and ChAT-IR in the myenteric plexus of the colon from healthy young adult mice

The myenteric plexus of the colon tissues were triple-labelled with β -Tubulin III, Netrin-1 and ChAT, and the images indicate labelling of β -Tubulin III and Netrin-1 (**A1-3**); β -Tubulin III and ChAT (**B1-3**); and, Netrin-1 and ChAT (**C1-3**). The inserted box areas of **C1-3** are shown with a high magnification view (**C1'-3'**). In **C1'-3'**, the filled arrowhead indicates the neuronal cell bodies that are strongly Netrin-1-positive, but ChAT-negative (N1++/ChAT-). The empty arrowhead in **C1'-3'** indicates the neuron that is Netrin-1-negative but strongly ChAT-positive (N1-/ChAT++). The filled arrow in **C1'-3'** indicates the neuron that is strongly Netrin-1-positive but also weakly positive for ChAT (N1++/ChAT+). The empty arrow in **C1'-3'** indicates the neuron that is weakly Netrin-1-positive but also strongly positive for ChAT (N1+/ChAT++). (Strongly positive: ++; weakly positive: +). Scale bar: 50 μm . The graph (**D**) indicates

the proportion of the immunoreactive neurons for N1⁺⁺/ChAT⁻, N1⁻/ChAT⁺⁺ and N1⁺(+)/ChAT⁺(+) were measured in the total area of 2mm². The population of N1⁺(+)/ChAT⁺(+) represents the populations of both N1⁺/ChAT⁺⁺ and N1⁺⁺/ChAT⁺. Data represented as mean \pm SD (n=3 animals).

Abbreviations: ChAT, choline acetyltransferase; N1, Netrin-1; +, weakly positive; ++, strongly positive; +(+) , both weakly and strongly positive; -, negative.

Figure 5. Co-localisation of Netrin-1-IR and nNOS-IR in the myenteric plexus of the colon from healthy young adult mice

The myenteric plexuses of the colon tissues were triple-labelled with β -Tubulin III, Netrin-1 and nNOS, and the images indicate labelling of β -Tubulin III and Netrin-1 (**A1-3**); β -Tubulin III and nNOS (**B1-3**); and, Netrin-1 and nNOS (**C1-3**). The inserted box areas of **C1-3** are shown with a high magnification view (**C1'-3'**). In **C1'-3'**, the filled arrowhead indicates a strong Netrin-1-immunoreactive neuron that was co-stained with nNOS (N1⁺⁺/nNOS⁺). The empty arrowhead in **C1'-3'**, indicates a weak Netrin-1-immunoreactive neuron that did not show nNOS-IR (N1⁺/nNOS⁻). (Strongly positive: N1⁺⁺; weakly positive: N1⁺). The arrow in **C2'** indicates a nNOS-positive neuron with flat lamella dendrites. This is known as a characteristic of mature nNOS enteric neurons. Scale bar: 50 μ m. The graph (**D**) indicates the proportion of the immunoreactive neurons for N1⁺⁺, nNOS⁺ and N1⁺⁺/nNOS⁺ were measured within the total area of 2mm². Data represented as mean \pm SD (n=3 animals).

Abbreviations: nNOS, neuronal nitric oxide synthase; N1, Netrin-1; +, weakly positive; ++, strongly positive; +(+) , either weakly or strongly positive; -, negative.

Supplementary Figure 1. Secondary antibody control experiment

The myenteric plexus of the colon tissues were labelled with either two primary antibodies for NF-H and Netrin-1 **(A)** or a single primary antibody for NF-H **(B)**; either NF-H and DCC **(C)** or NF-H alone **(D)**. Corresponding species-specific secondary antibodies were applied to the tissues. No binding of Netrin-1 or DCC was found when anti-Netrin-1 or anti-DCC antibody was omitted, and only secondary antibodies were applied. Scale bar: 25 μm .

Supplementary Figure 2. Absorption control experiment

The myenteric plexus of the colon tissues were incubated with anti-NF-H antibody and preabsorbed Netrin-1 antibody, followed by incubation with corresponding secondary antibodies. The red channel showed a low level of non-specific binding around neuronal ganglions **(A)**, while the green channel exhibited a specific binding of NF-H **(B)** in the myenteric plexus. Double-labelling of NF-H and Netrin-1 **(C)** was conducted parallel to the absorption control. Scale bar: 25 μm .

Supplementary Figure 3. DCC antibody specificity

SW480 cells were transfected with plasmids including Neo-Bam (empty vector plasmid), DCC-Mutant (DCC gene missing entire intracytoplasmic domain), and DCC-Full (DCC gene full length). A representative Western blot image **(A)** shows the DCC immunoblot on the cells transfected with full length of DCC plasmid, but not on DCC-Mutant. The epitope region (C-terminus) of DCC antibody used in this study was subject to Basic Local Alignment Search Tool (BLAST) **(B)**. The intracellular

domain (amino acids 1375-1425 of DCC of human origin, accession # P43146) was searched, and showed 10 protein sequences that are found to be aligned within the C-terminus of DCC. Among those BLAST hits, only 2 protein sequences exhibited significant protein sequence alignments with the intracellular domain of DCC, and they are DCC isoform and DCC precursor.

Supplementary Figure 4. Netrin-1-like and DCC immunoreactivity in the myenteric plexus of the colon from healthy young adult mice

(A) The myenteric plexus of the colon tissues were stained with DAPI, nuclei staining, and Netrin-1 using a chicken polyclonal anti-Netrin-1 antibody (Aves Labs, Cat #: NET). **(B)** The myenteric plexus was also labelled with a neuronal marker, PGP9.5 and DCC using a rabbit polyclonal anti-DCC antibody (Abnova, Cat #: PAB17539). The merged image demonstrates the co-localisation of enteric ganglia with DCC receptors. N=3 animals. Scale bars: 25 μ m.

Abbreviations: DCC, deleted in colorectal cancer; PGP9.5, protein gene product 9.5; DAPI, 4',6-diamidino-2-phenylindole.

Tables

Table 1. List of primary antibodies used in immunofluorescence assays

Primary antibody	Host species	Source	Catalogue number	Dilution
PGP9.5	Rabbit	Merck Millipore	AB1761-I	1:1000
DCC	Goat	Santa Cruz (A-20)	Sc-6535	1:500
Netrin-1	Rabbit	Santa Cruz (H-104)	Sc-20786	1:500
β -Tubulin III	Chicken	Abcam	Ab107216	1:1000
ChAT	Goat	Merck Millipore	AB144P	1:500
nNOS	Goat	Novous Biologicals	NB100-858	1:500
GFAP	Rabbit/Goat	Abcam	ab7260/ab53554	1:2000

Table 2. List of secondary antibodies used in immunofluorescence assays

Secondary antibody	Source	Catalogue number	Dilution
Donkey anti-goat rhodamine	Jackson ImmunoResearch Laboratories	705-295-147	1:300
Donkey anti-mouse FITC	Merck Millipore	AP182F	1:300
Donkey anti-rabbit rhodamine	Jackson ImmunoResearch Laboratories	AP182R	1:300
Donkey anti-rabbit FITC	Merck Millipore	AP182F	1:300
Donkey anti-goat FITC	Merck Millipore	AP180F	1:300
Donkey anti-chicken FITC	Merck Millipore	AP194F	1:300

Figure 1

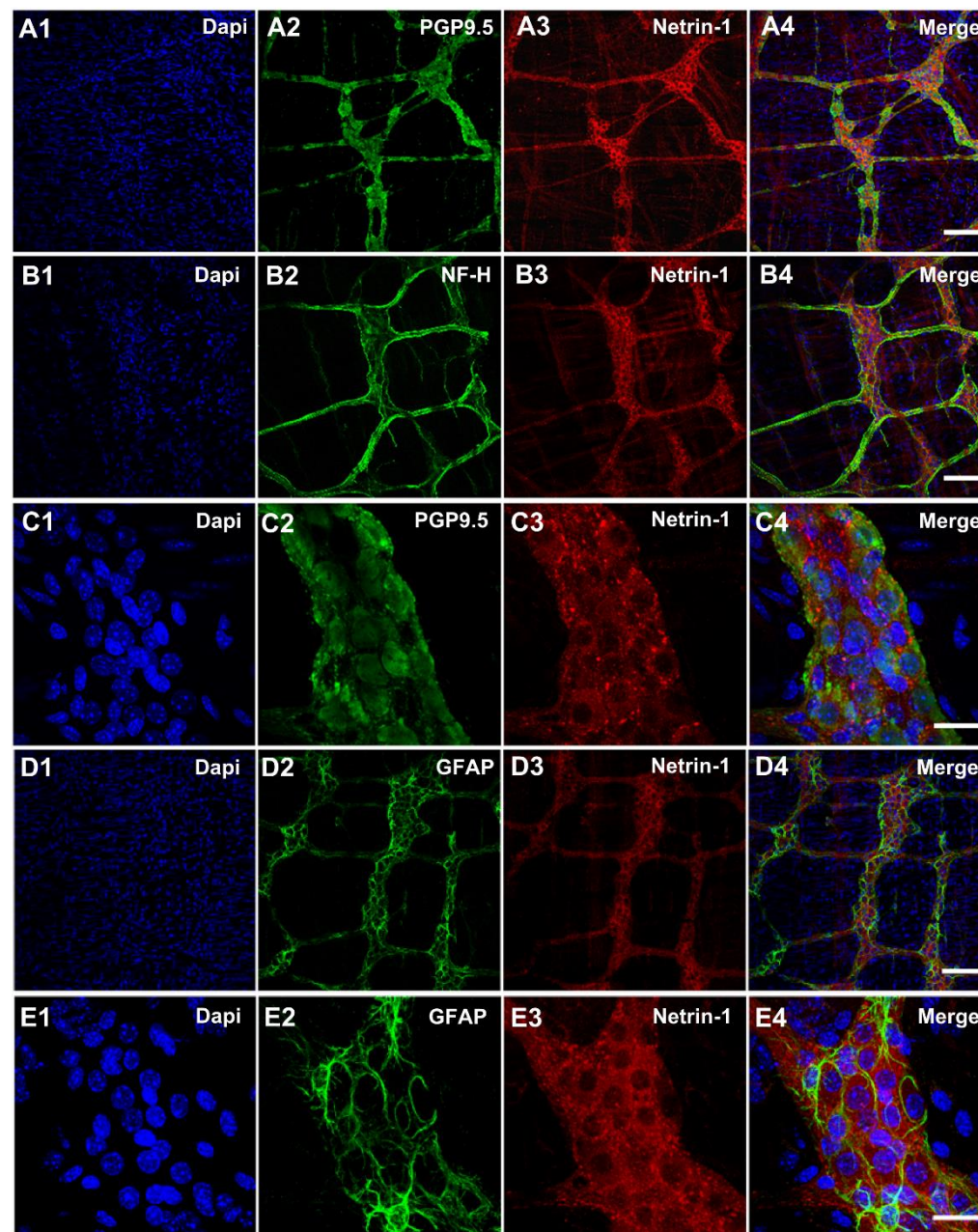


Figure 2

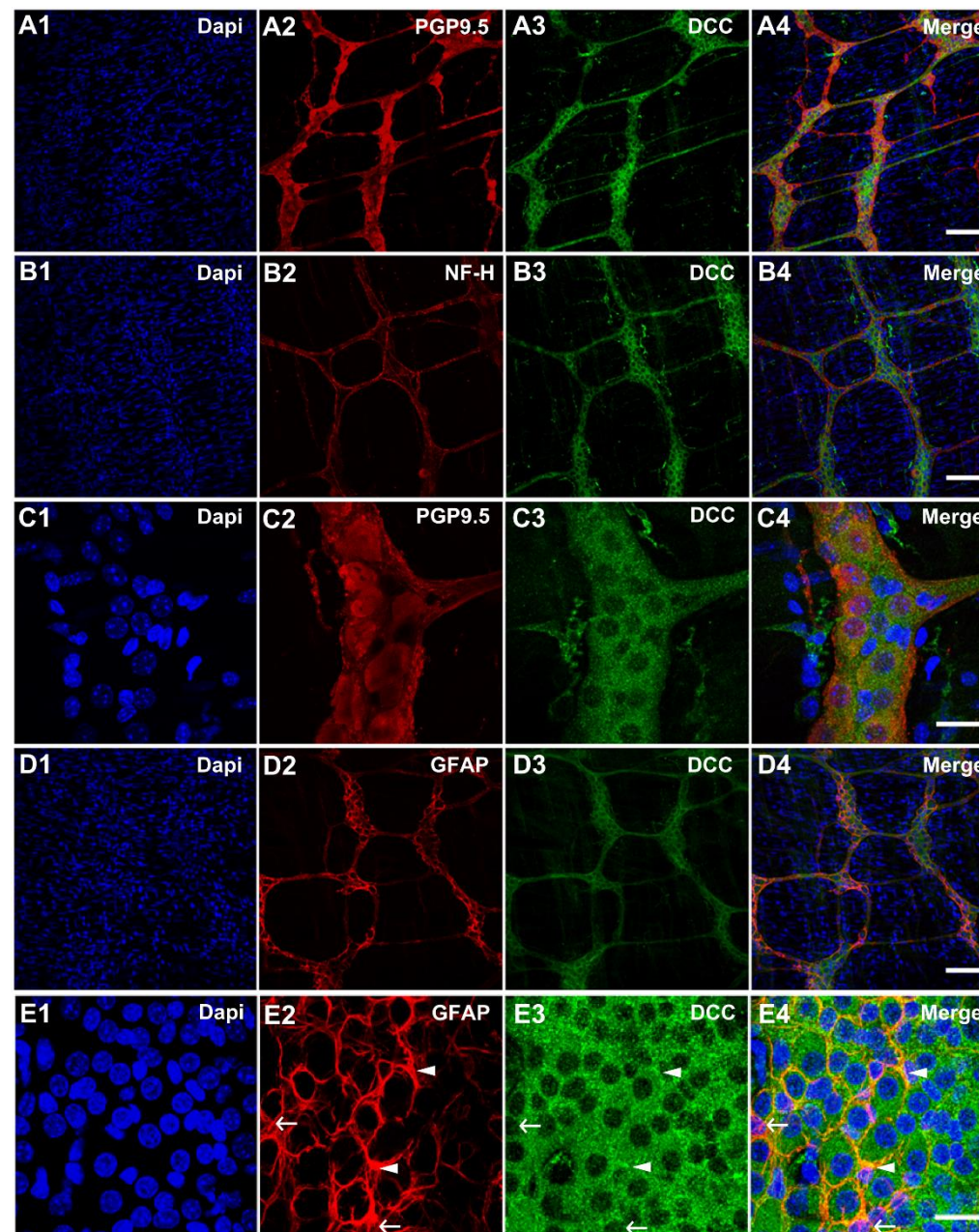


Figure 3

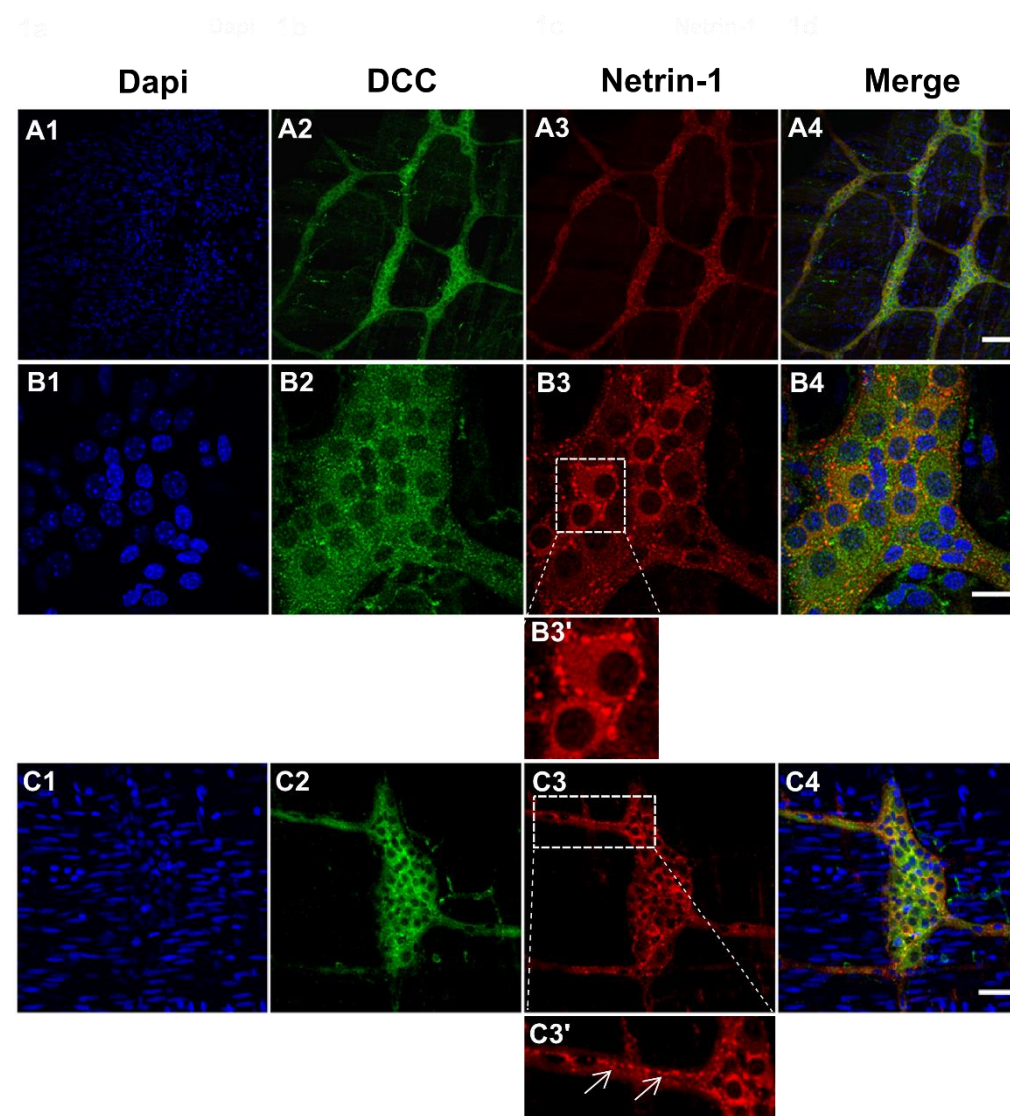


Figure 4

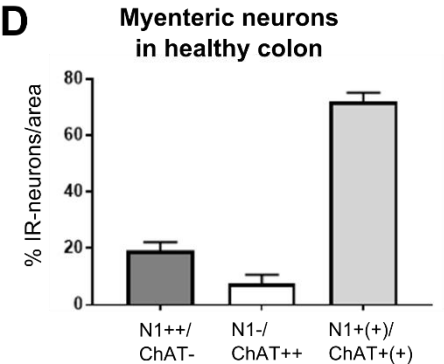
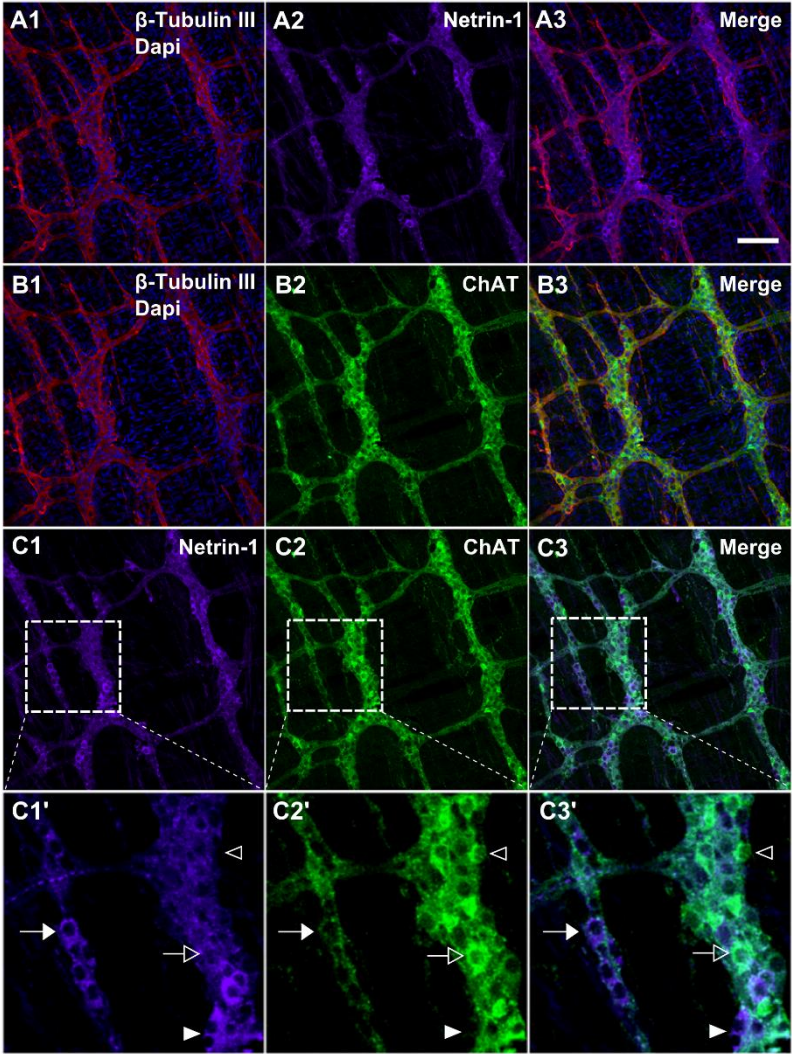


Figure 5

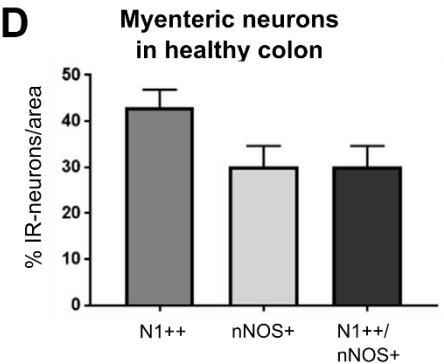
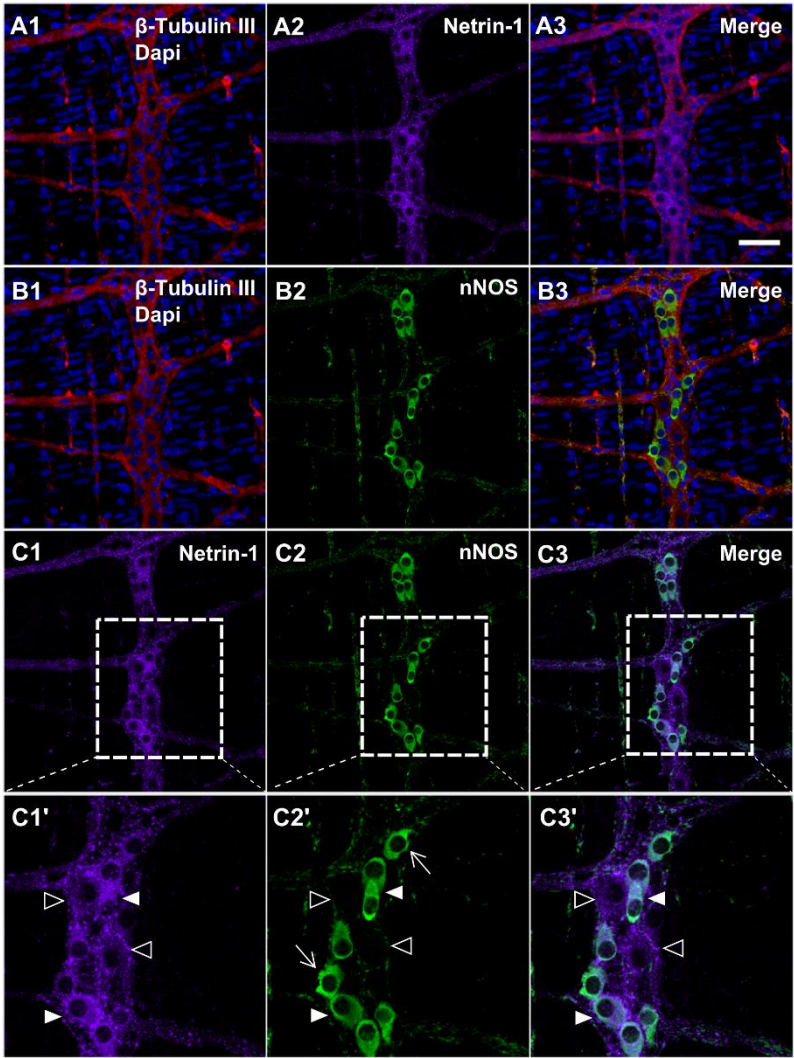


Figure SF1

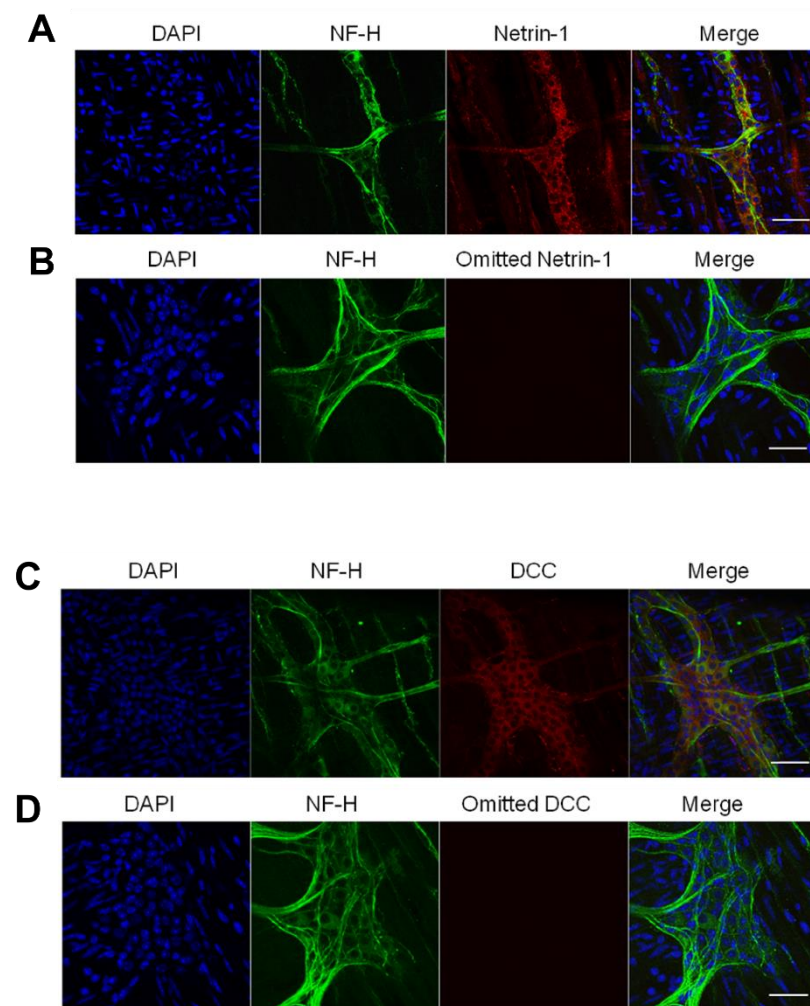


Figure SF2

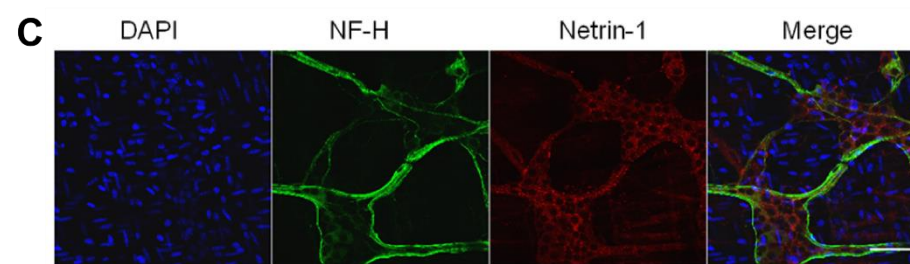
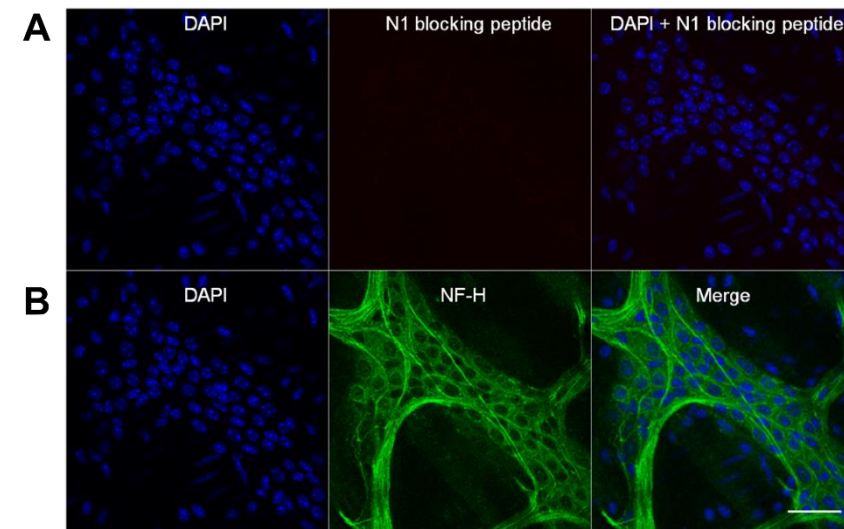
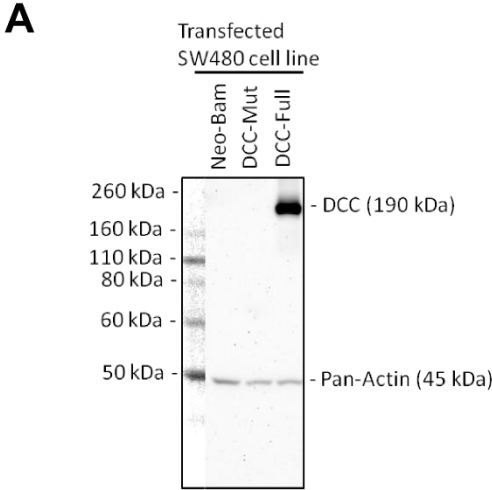


Figure SF3



B NCBI BLAST: Protein Sequence
Distribution of 10 Blast Hits on the Query Sequence

Descriptions

Sequences producing significant alignments:

Description	Max score	Total score	Query cover	E value	Ident	Accession
PREDICTED: netrin receptor DCC isoform X1 [Mus musculus]	97.1	97.1	100%	3e-24	96%	XP_006525651.1
netrin receptor DCC precursor [Mus musculus]	97.1	97.1	100%	3e-24	96%	NP_031857.2
PREDICTED: neogenin isoform X5 [Mus musculus]	44.3	44.3	94%	5e-06	53%	XP_006510909.1
PREDICTED: neogenin isoform X2 [Mus musculus]	44.3	44.3	94%	5e-06	53%	XP_006510906.1
neogenin isoform 2 precursor [Mus musculus]	44.3	44.3	94%	5e-06	53%	NP_001036217.1
PREDICTED: neogenin isoform X6 [Mus musculus]	44.3	44.3	94%	5e-06	53%	XP_006510910.1
PREDICTED: neogenin isoform X1 [Mus musculus]	44.3	44.3	94%	5e-06	53%	XP_006510905.1
neogenin isoform 1 precursor [Mus musculus]	44.3	44.3	94%	5e-06	53%	NP_032710.2
PREDICTED: neogenin isoform X4 [Mus musculus]	44.3	44.3	94%	5e-06	53%	XP_006510908.1
PREDICTED: neogenin isoform X3 [Mus musculus]	44.3	44.3	94%	5e-06	53%	XP_006510907.1

Figure SF4

